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14. ABSTRACT Mutational inactivation of BRCA1 confers a cumulative lifetime risk of breast and ovarian cancers. However, the underlying basis for the tissue-specific tumor suppressor function of BRCA1 remains poorly defined. Previously, we described a novel function for BRCA1 in suppressing the ligand-independent transcriptional activity of the estrogen receptor α (ER α), a principal determinant of the growth and differentiation of breasts and ovaries. Based on these observations, we hypothesized that BRCA1 represents a ligand-reversible barrier to transcriptional activation by unliganded ER α and, further, that mutational inactivation of BRCA1 promotes epithelial cell proliferation through aberrant expression of estrogen-responsive genes, possibly contributing to tumorigenesis. To substantiate this hypothesis we have proposed the following aims: (1) to biochemically reconstitute BRCA1-mediated ligand-independent repression of ER α in vitro; (2) to examine the role of estrogen-induced site-specific BRCA1 phosphorylation in the regulation of BRCA1-mediated ligand-independent ER α repression; and (3) to determine the role of BRCA1 in the control of paracrine growth signaling in the breast. Collectively, these studies should reveal novel insight into the tissue-specific tumor suppressor function of BRCA1 and provide defined molecular targets for future intervention in breast cancer.					
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INTRODUCTION

Mutational inactivation of the BReast CAncer susceptibility gene product, BRCA1, confers a cumulative lifetime risk of breast and ovarian cancers (1,2). However, the underlying basis for the tissue-specific tumor suppressor properties of BRCA1 remains poorly defined. Previously, we described a novel function for BRCA1 in suppressing the ligand-independent transcriptional activity of the estrogen receptor α (ER α), a principal determinant of the growth and differentiation of breasts and ovaries (3). Importantly, we documented that clinically validated BRCA1 missense mutations abrogate this repression activity, thereby suggesting that its ER α specific repression function is important for the biological activity of BRCA1 in breast and ovarian tumor suppression. In human breast cancer cells, we observed an association between BRCA1 and ER α at endogenous estrogen-responsive gene promoters before, but not after, estrogen stimulation. Furthermore, we demonstrated that attenuation of BRCA1 expression in estrogen dependent human ovarian cancer cells could be correlated with increases in both the estrogen-independent transcription of ER α -target genes and estrogen-independent cellular proliferation. Based on these observations, we hypothesized that BRCA1 represents a ligand-reversible barrier to transcriptional activation by unliganded ER α and, further, that mutational inactivation of BRCA1 promotes breast epithelial cell proliferation through aberrant expression of estrogen responsive genes, possibly contributing to tumorigenesis. To substantiate this hypothesis we proposed: (1) to biochemically reconstitute BRCA1-mediated ligand-independent repression of ER α *in vitro*; (2) to examine the role of estrogen induced site-specific BRCA1 phosphorylation in the regulation of BRCA1-mediated ligand-independent ER α repression; and (3) to determine the role of BRCA1 in the control of paracrine growth signaling in the breast. These studies should reveal novel insight concerning how mutational inactivation of a ubiquitously expressed tumor suppressor could have restricted consequences in the breast and ovary. Furthermore, we expect these studies to have important implications with respect to the future treatment of breast cancer. Mechanistic insight into the biological role and regulation of BRCA1 as a repressor of ER α function should expedite the development of tissue-specific chemotherapeutic approaches intended to restore an appropriate hormonal response to BRCA1-mutant breast epithelial cells.

Body

Technical Objective 1. To biochemically reconstitute BRCA1-mediated ligand-independent repression of estrogen receptor α (ER α) *in vitro* from purified components.

Task 1: To reconstitute estrogen-independent ER α -directed transcriptional activation *in vitro*.

Our plan was to use nuclear extracts derived from *Brcal-1*- mouse embryo fibroblasts (MEFs) and purified recombinantly expressed ER α on chromatin-assembled templates *in vitro*. In our prior progress reports, we described the biochemical reconstitution of estrogen-independent ER α -directed transcriptional activation using nuclear extracts derived from *Brcal-1*-mouse embryo fibroblasts (MEFs) and highly purified recombinant ER α on an ER α responsive reporter template *in vitro*. However, the relatively weak activation potential of ER α in this system prompted us to explore an alternative approach to achieve more robust activation from which to observe the ability of recombinant BRCA1 protein to repress ligand-independent ER α -directed transcriptional activation. This alternative approach relied on the use of a hybrid VP16-GAL4-ER α transactivator that bears the ER α ligand-binding domain translationally fused to a constitutively potent VP16-GAL4 hybrid activator. Previously, we showed that the ER α ligand-binding domain converts VP16-GAL4 from a constitutive to an estrogen-dependent transactivator in *Brcal-1*-proficient, but not in *Brcal-1*-deficient MEFs, thus revealing the ER α

ligand-binding domain to be a platform for the recruitment of BRCA1 from which the latter may confer ligand-independent repression on a linked activation domain (3). Because VP16-GAL4-ER α carries a potent transactivation domain whose constitutive activity is repressed by recruitment of BRCA1 through the ER α hormone-binding domain, we proposed to use this hybrid transactivator as an alternative approach to reconstitute BRCA1-mediated ligand-independent repression of ER α activity. To this end, we generated and expressed a recombinant six histidine-tagged-VP16-GAL4-ER α in *E. coli*. However, our exhaustive attempts to purify this hybrid transactivator in soluble form proved to be futile, thus forcing us to revisit our original transcription system that relies on the use of recombinant full-length ER α protein. In fact, this system is preferable for study since it represents a more physiologically faithful system to study BRCA1-mediated repression of full-length ER α activity. Reasoning that the low activation potential of unliganded ER α in our reconstituted transcription system could derive from limiting amounts of putative ER α co-activator that is reduced or absent in *Brcal-1*-MEFs, we undertook to identify ligand-independent co-activators of ER α from human cells. Once identified, this activity could be used to supplement nuclear extracts from *Brcal-1*-MEFs in order to achieve robust ER α -directed transcription *in vitro* and thereby provide a suitable starting point from which to study BRCA1-mediated repression of unliganded ER α transcriptional activity.

To facilitate the identification of unliganded ER α -associated proteins and therefore possible co-regulators of ER α , we used retroviral-mediated gene transfer to engineer a HeLaS3 cervical carcinoma-derived cell line (fER α /S3) that stably expresses a FLAG epitope-tagged ER α . To isolate ligand-independent ER α -associated proteins, we subjected partially purified protein fractions from hormone-deprived fS3/ER α on an anti-FLAG M2 monoclonal antibody affinity column. This procedure resulted in the specific isolation of fER α along with ~23 fER α -associated polypeptides (ERAPs) ranging in size from 35 to 250 kDa (supplemental Fig. 1 of Appendix 1). Mass spectrometric based-peptide sequence analysis performed by the Harvard Microchemistry facility revealed the identity of one ERAP to be the product of the *Deleted in Breast Cancer-1* gene, DBC-1 (4). The gene encoding DBC-1 was originally identified based on its localization to chromosome 8p21, a region frequently deleted in breast cancers (4). However, refined deletion analysis within this region revealed a second gene, *deleted in breast cancer 2* (DBC-2), to encode a likely breast tumor suppressor, and confirmed that *DBC-1* expression is not substantially extinguished in cancers from any source (4). In fact, a search of the Oncomine database revealed DBC-1 to be statistically significantly upregulated in breast carcinoma versus normal breast tissue as well as breast ductal carcinoma versus other cancers (5, 6). Furthermore, DBC-1 was found in three independent studies totaling 369 breast tumor samples to be statistically significantly overexpressed in ER α -positive versus ER α -negative breast tumors (7, 8, 9). Based on our identification of DBC-1 as a ligand-independent ER α -interacting protein as well as its provocative expression profile in breast cancers, we therefore undertook to explore the physical basis, biological regulation, and functional consequence of the interaction between DBC-1 and ER α in human breast cancer cells. Below, our published findings in this regard are summarized.

First, the DBC-1 amino terminus binds directly to the ER α hormone-binding domain both *in vitro* and in human breast cancer cells in a strict ligand-independent manner (Figs. 1-3 of Appendix 1). Second, like E2, the antiestrogens tamoxifen and ICI 182,780 disrupt the DBC-1/ER α interaction in human breast cancer cells, thus revealing the DBC-1/ER α interface to be an unanticipated target of endocrine compounds commonly used in hormonal therapy (Fig. 4 of Appendix 1). Third, DBC-1 depletion reduces the steady-state level of unliganded, but not liganded, ER α protein through specific inhibition of ER α protein synthesis (Fig. 5 of Appendix 1 and data not shown). Fourth, DBC-1 depletion inhibits estrogen-independent proliferation (Fig. 6 of Appendix 1) and promotes estrogen-independent apoptosis (Fig. 7 of Appendix 1) of ER α -

positive, but not ER α -negative, breast cancer cells in a manner reversible by endocrine agents that either disrupt the DBC-1/ER α complex (E2) or that reduce the level of ER α (ICI 182,780). Together, these preliminary findings establish a principal biological function for DBC-1 in the modulation of ER α expression and hormone-independent breast cancer cell survival. These findings were published in 2007 (Trauernicht et al. 2007. Modulation of estrogen receptor α protein level and survival function by DBC-1. Mol. Endocrinol. 21: 1526-1536 – Appendix 1).

Our finding that DBC-1 functions as a hormone-independent prosurvival factor in human breast cancer cells prompted us to examine its possible role in endocrine resistance. To this end, we examined the expression and function of DBC-1 in a three-stage MCF-7 cell-based model of acquired endocrine resistant breast cancer (10). This model system is based on the ER α -positive MCF-7 human breast cancer cell line, which is estrogen-dependent for growth and sensitive to the growth inhibitory actions of antiestrogens, including the selective estrogen receptor modulator (SERM) tamoxifen and the selective estrogen receptor downregulator (SERD) ICI 182,780 (faslodex, fulvestrant). Long-term passage of MCF-7 tumor xenografts in ovariectomized mice led to derivation of the MCF-7/LCC1 (LCC1) cell line, which is estrogen-independent but antiestrogen-sensitive. Subsequent long-term culture of LCC1 cells *in vitro* in the presence of ICI 182,780 produced the MCF7/LCC9 (LCC9) cell line, which is fully resistant to both estrogen and ICI 182,780, and cross-resistant to tamoxifen. This model system, derived through stepwise selection of MCF-7 cells first to a low estrogen environment *in vivo* followed by long-term culture in the presence of an antiestrogen, mimics a clinical scenario [Phase II endocrine resistance (11)] in which breast cancer patients undergo exhaustive hormonal therapy (first-line treatment with an aromatase inhibitor followed by second-line treatment with an antiestrogen) leading to the acquisition of a fully estrogen-independent and antiestrogen-resistant tumor phenotype. Using this three-stage MCF-7 cell-based model of acquired endocrine resistant breast cancer, we found that DBC-1 is upregulated during the acquisition of endocrine resistance and, further, that targeted suppression of DBC-1 triggers a rapid and profound apoptotic response in endocrine resistant LCC1 and LCC9 breast cancer cells (Fig. 1 of Appendix 2). Together, these findings establish DBC-1 as a critical and heretofore unknown determinant of endocrine resistant breast cancer cell survival. These findings have since been published (Trauernicht et al. 2010. DBC-1 mediates endocrine resistant breast cancer cell survival. Cell Cycle 9: 1218-1219 – Appendix 2).

Technical Objective 2. To examine the role of estrogen-induced site-specific BRCA1 phosphorylation in the regulation of BRCA1-mediated ligand-independent ER α repression.

Our plan was to immunoprecipitate BRCA1 from hormone-depleted MCF-7 human breast cancer cells stimulated with estrogen followed by both mass spectrometric analysis and immunoblot analysis using phosphopeptide-specific BRCA1 antibodies. However, we were unable to obtain sufficient quantities of immunoprecipitated BRCA1 protein for mass spectrometric-based identification.

Technical Objective 3. To determine the role of BRCA1 in the control of paracrine growth signaling in the breast.

Our original plan was to compare the ability of conditioned serum-free medium obtained from *Brcal* +/– and *Brcal*-1– murine mammary epithelial cells cultured in the absence or in the presence of estrogen for their potential to promote the growth of ER-negative MCF10A mammary epithelial cells in culture. However, we encountered intractable difficulties in establishing primary cultures of mammary epithelial cells from *Brcal* +/– and *Brcal*-1– mice, as proposed in our application. In order to circumvent these difficulties yet adhere to our original

aim, we adopted an alternative and much simpler strategy to achieve the conditional inactivation of BRCA1 in culture. Our strategy was to establish clonal T47-D breast cancer cell lines supporting conditional (tetracycline inducible) knockdown of BRCA1 using shRNAs. We succeeded in identifying a BRCA1-specific shRNA that reduced BRCA1 levels by ~90% in T47-D cells. However, we experienced intractable difficulties in obtaining clonal lines of these knockdown cells. Similarly, our attempts to circumvent this issue by the use of retroviral gene transfer also met with little success.

KEY RESEARCH ACCOMPLISHMENTS (REPORTED YEAR ACCOMPLISHMENTS IN BOLD)

- ◆ Expression in and purification of FLAG epitope-tagged human estrogen receptor (ER α) from insect Sf21 cells.
- ◆ Biochemical reconstitution of estrogen-independent ER α -directed transcriptional activation using nuclear extracts from *Brca1*^{-/-} mouse embryo fibroblasts (MEFs) and highly purified recombinant fER α on an ER α -responsive reporter template in vitro.
- ◆ Construction and expression in *E. coli* of a recombinant VP16-GAL4- ER α hybrid transactivator protein for analysis of constitutive activity in *Brca1*^{-/-} MEF nuclear extract.
- ◆ Identification of the product of the deleted in breast cancer 1 gene, DBC-1, as an unliganded ER α -associated protein.
- ◆ Identification of DBC1 as a steroid hormone selective co-activator.
- ◆ Experimental validation of BRCA1 knockdown in T47-D human breast cancer cells.
- ◆ **Established a principal biological function for DBC-1 in the modulation of ER α expression and hormone-independent breast cancer cell survival.**
- ◆ **Established DBC-1 as a critical and heretofore unknown determinant of endocrine resistant breast cancer cell survival.**

REPORTABLE OUTCOMES TO DATE

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CONCLUSIONS

We have identified principal biological functions for the deleted in breast cancer gene product, DBC-1, as a hormone selective ER α co-activator, a key regulator of ER α expression and hormone-independent breast cancer cell survival, and a critical and heretofore unknown determinant of endocrine resistant breast cancer cell survival.

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Modulation of Estrogen Receptor α Protein Level and Survival Function by DBC-1

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Acquired resistance to endocrine therapy represents a major clinical obstacle to the successful management of estrogen-dependent breast cancers expressing estrogen receptor α (ER α). Because a switch from ligand-dependent to ligand-independent activation of ER α -regulated breast cancer cell growth and survival may define a path to endocrine resistance, enhanced mechanistic insight concerning the ligand-independent fate and function of ER α , including a more complete inventory of its ligand-independent cofactors, could identify novel markers of endocrine resistance and possible targets for therapeutic intervention in breast cancer. Here, we identify the deleted in breast cancer 1 gene product DBC-1 (KIAA1967) to be a principal determinant of unliganded ER α expression and survival function in human breast cancer cells. The DBC-1 amino terminus binds directly to the ER α hormone-binding domain both *in vitro* and *in vivo* in a strict ligand-independent manner. Furthermore, like estrogen, the

antiestrogens tamoxifen and ICI 182,780 (7 α ,17 β -[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol) disrupt the DBC-1/ER α interaction, thus revealing the DBC-1/ER α interface to be a heretofore-unrecognized target of endocrine compounds commonly used in hormonal therapy. Notably, RNA interference-mediated DBC-1 depletion reduces the steady-state level of unliganded but not liganded ER α protein, suggesting that DBC-1 may stabilize unliganded ER α by virtue of their direct association. Finally, DBC-1 depletion promotes hormone-independent apoptosis of ER α -positive, but not ER α -negative, breast cancer cells in a manner reversible by endocrine agents that disrupt the DBC-1/ER α interaction. Collectively, these findings establish a principal biological function for DBC-1 in the modulation of ER α expression and hormone-independent breast cancer cell survival. (*Molecular Endocrinology* 21: 1526–1536, 2007)

BREAST CANCER IS the leading cause of death among American women between the ages of 20 and 59 yr (1). Among a variety of established etiologic factors linked to breast cancer, the steroid hormone estrogen [17- β -estradiol (E2)] has long been implicated in disease pathogenesis. Numerous animal studies have revealed that E2 can induce and promote breast cancer, whereas estrogen ablation therapy or the administration of antiestrogens can oppose these effects (2–4). The physiological effects of E2 in the breast are mediated by cognate receptors that are expressed as two structurally related subtypes, estrogen receptor α (ER α) and β (ER β) (5–8). ER α is the predominant receptor isoform expressed in breast cancer cells, and approximately 70% of breast cancer patients score positive for ER α at diagnosis (9–12).

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Abbreviations: CYP40, Cyclophilin 40; E2, 17- β -estradiol; ER, estrogen receptor; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; HSP90, heat shock protein 90; NF- κ B, nuclear factor κ B; NP-40, Nonidet P-40; RNAi, RNA interference; siRNA, small interfering RNA.

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ER α is therefore a dominant etiologic and valuable predictive factor with respect to breast cancer development and hormone sensitivity status. Endocrine therapy, which seeks to block ER-mediated mitogenic signaling, has emerged as one of the most important systemic therapies in breast cancer management; however, therapeutic resistance, either inherent (*de novo* resistance) or acquired during treatment (acquired resistance) remains a significant clinical roadblock to effective disease management (13).

Although *de novo* resistance to endocrine therapy derives primarily from loss of ER α expression, the biological mechanism underlying acquired endocrine resistance is incompletely understood and almost certainly multifactorial in nature (14, 15). Nonetheless, the emergence of endocrine resistance is often coincident with a shift from ligand-dependent to ligand-independent control of ER α -regulated breast cancer cell growth and survival, possibly reflecting bidirectional molecular crosstalk between ER α and growth factor signaling pathways (14, 16, 17). Because ligand-independent activation of ER α may therefore define a path to endocrine resistance, enhanced mechanistic insight concerning the ligand-independent function and regulation of ER α , including a more complete inventory of its ligand-independent cofactors, could identify novel prognostic markers of endocrine resistance and possible

targets for therapeutic intervention in breast cancer. Toward this objective, we have undertaken a proteomics-based approach to isolate ligand-independent ER α protein interaction networks. Herein, we identify the deleted in breast cancer-1 gene product DBC-1 (KIAA1967) to be a direct ligand-independent binding partner of ER α . Functional analyses further reveal DBC-1 to be a principal determinant of unliganded ER α protein levels and survival activity in human breast cancer cells.

The gene encoding DBC-1 was originally identified during a genetic search for candidate breast tumor suppressor genes on a human chromosome 8p21 region frequently deleted in breast cancers. However, refined deletion analysis within this region revealed a second gene, deleted in breast cancer 2 (*DBC-2*), to encode a likely breast tumor suppressor, and further confirmed that *DBC-1* expression is not substantially extinguished in cancers from any source (18). In fact, a search of the Oncomine database of published cancer microarray data (www.oncomine.org), which currently permits analysis of gene expression data derived from 132 DNA microarray datasets among 24 different cancer types, reveals DBC-1 to be statistically significantly upregulated in breast carcinoma vs. normal breast tissue as well as breast ductal carcinoma vs. other cancers (19, 20). Furthermore, DBC-1 was found in three independent studies totaling 369 breast tumor samples to be statistically significantly overexpressed in ER-positive vs. ER-negative breast tumors (21–23).

Little is currently known regarding the molecular and cellular function of DBC-1 in breast or other tissues. Recently, DBC-1 was linked physically to the TNF- α /nuclear factor κ B (NF- κ B) pathway by proteomic analysis (24), whereas caspase-dependent processing of DBC-1 early in apoptosis induced by diverse stimuli, including TNF- α , was shown to unmask a proapoptotic function for the DBC-1 carboxyl terminus in the cytosol of moribund cells (25). However, full-length DBC-1 is predominantly localized to the nucleus of healthy cells (25), and its normal biological function therein has heretofore remained unknown. Based on our identification of DBC-1 as a ligand-independent ER α -interacting protein as well as its provocative expression profile in breast cancers, we therefore undertook to explore the physical basis, biological regulation, and functional consequence of the interaction between DBC-1 and ER α in human breast cancer cells. Our findings reveal that the DBC-1 amino terminus binds directly to the ER α hormone-binding domain both *in vitro* and *in vivo* in a strict E2-independent manner. Furthermore, like E2, the antiestrogens tamoxifen and ICI 162,780 (7 α ,17 β -[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol) disrupt the DBC-1/ER α interaction, thus revealing the DBC-1/ER α interface to be an unanticipated target of these endocrine compounds. Finally, DBC-1, in a manner dependent on direct interaction with ER α , suppresses breast cancer cell apoptosis in the absence of hormone. These findings thus establish

a principal biological function for DBC-1 in the modulation of ER α expression and survival activity and further identify DBC-1 as a possible endocrine response determinant and potential therapeutic target in breast cancer.

RESULTS

DBC-1 Interacts with ER α *in Vivo* in a Ligand-Independent Manner

During the course of a targeted search for ligand-independent ER α interaction partners, we identified DBC-1 by mass spectrometric-based peptide sequence analysis of proteins coimmunoprecipitated specifically with unliganded, but not liganded, ER α (supplemental Fig. 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>). To validate the ligand-independent interaction between DBC-1 and ER α *in vivo*, we used a mammalian two-hybrid interaction analysis. Chimeric proteins consisting of DBC-1 fused to the GAL4 DNA-binding domain and ER α fused to the VP16 activation domain were expressed with or without one another in HeLa cells and examined for their respective abilities to activate transcription from a reporter template controlled by GAL4 DNA-binding sites in both the absence and presence of E2. In the absence of E2, DBC-1 and ER α exhibited a robust interaction that was disrupted by addition of E2 to the cell culture medium (Fig. 1A). Additional analysis of DBC-1 amino and carboxyl truncation derivatives revealed that the ligand-independent association between DBC-1 and ER α is mediated entirely by the amino-terminal half of DBC-1 (Fig. 1B).

To confirm the ligand-independent *in vivo* association between DBC-1 and ER α using a more biologically relevant approach, we examined the ability of antibodies specific for ER α or DBC-1 to coprecipitate one another in MCF-7 human breast cancer cells, which express both ER α and DBC-1. This analysis revealed that DBC-1 was specifically and reciprocally coimmunoprecipitated along with unliganded, but not liganded, ER α , demonstrating that the two endogenous proteins interact in a strict ligand-independent manner in human breast cancer cells (Fig. 1C). We also confirmed a ligand-independent interaction between endogenous DBC-1 and ER α in both T-47D human breast and BG-1 human ovarian cancer cell lines, thus revealing the DBC-1/ER α interaction to be conserved in a variety of ER α -expressing cell lines (Fig. 1D).

Heat shock protein 90 (HSP90) together with additional heat shock family members and immunophilins are known to form a heteromeric chaperone complex that sequesters neosynthesized and unliganded ER α in an inactive state, primes it for ligand binding, and protects it from proteolytic degradation (26–28). We initially examined the physical relationship between unliganded ER α in complex with HSP90-based chap-

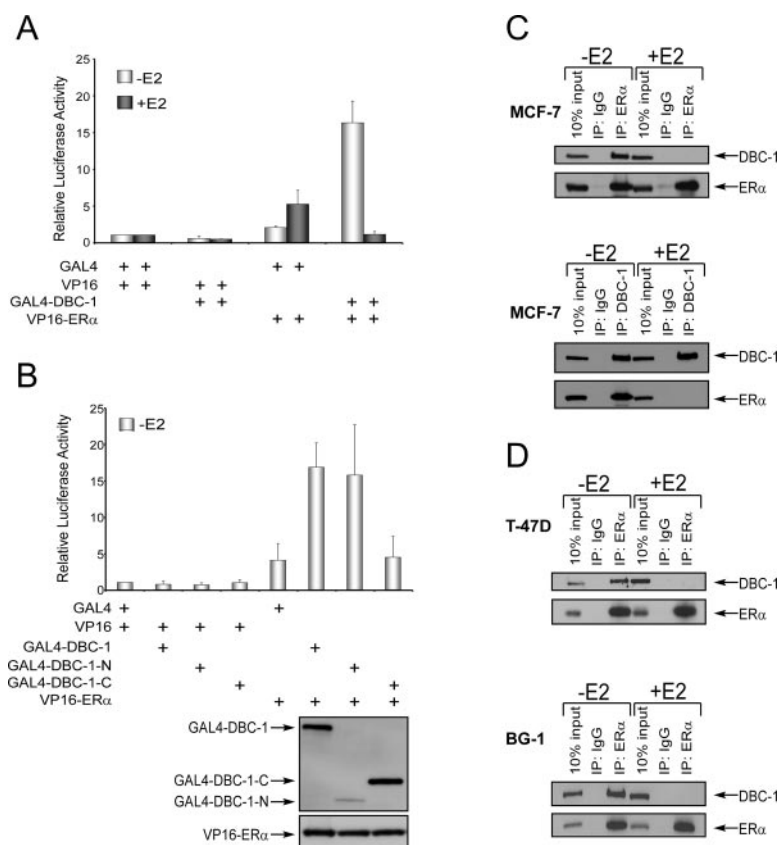


Fig. 1. DBC-1 and ER α interact *In Vivo* in a Ligand-Independent Manner

A, B, Mammalian two-hybrid interaction analysis. A, HeLa cells cultured in hormone-free medium for 3 d were transfected with the indicated combinations of mammalian expression plasmids encoding the yeast GAL4 DNA-binding domain (GAL4), the *Herpes simplex* virus VP16 transactivation domain (VP16), a GAL4-DBC-1 chimera, and a VP16-ER α chimera. Twenty-four hours after transfection, cells were treated without (–E2) or with (+E2) E2 (10^{-7} M) for an additional 24 h before cell harvest and assay of transfected whole-cell lysates for luciferase activity produced from a cotransfected GAL4 DNA-binding site driven-reporter template. Luciferase values are expressed relative to the luciferase activity obtained in cells transfected with both the GAL4 and VP16 expression vectors, which was arbitrarily assigned a value of 1. Luciferase activities were first normalized to β -galactosidase activity obtained by cotransfection of a β -galactosidase expression vector. Error bars represent the SD from the average of at least three independent transfections performed in duplicate. Note that estrogen abolishes the interaction between GAL4-DBC-1 and VP16-ER α . B, Top, HeLa cells cultured for 3 d in hormone-free medium (–E2) were transfected with the indicated combinations of mammalian expression plasmids encoding GAL4, VP16, a GAL4-DBC-1 N-terminal chimera (amino acids 1–478), a GAL4-DBC-1 C-terminal chimera (amino acids 479–923), and a VP16-ER α chimera. Forty-eight hours after transfection, cells were harvested, and transfected whole-cell lysates were assayed for luciferase activity produced from a cotransfected GAL4 DNA-binding site driven-reporter template as described in A. Note that ER α interacts exclusively with the N terminus of DBC-1. Bottom, Harvested whole-cell lysates were resolved by SDS-12% PAGE and processed by immunoblot analysis with antibodies specific for GAL4-DBD or ER α as indicated by arrows. Note that differences in the relative expression levels of the GAL4-DBC-1 chimeras cannot explain differences in their respective ER α -binding capabilities. Results are representative of at least three independent experiments. C, D, Coimmunoprecipitation analysis. C, MCF-7 cells cultured in hormone-free medium for 3 d were treated without (–E2) or with (+E2) E2 (10^{-7} M) for 1 h before cell harvest and immunoprecipitation (IP) of whole-cell lysates with antibodies specific for ER α (top) or DBC-1 (bottom). Immunoprecipitates were resolved by SDS-10% PAGE and processed by immunoblot analysis using antibodies specific for DBC-1 or ER α as indicated by arrows. Note specific immunoprecipitation of DBC-1 by ER α -specific antibodies and ER α by DBC-1-specific antibodies only in the absence, but not in the presence, of estrogen. Results are representative of at least three independent experiments. D, T-47D (top) and BG-1 (bottom) cells cultured in hormone-free medium for 3 d were treated without (–E2) or with (+E2) E2 (10^{-7} M) for 1 h before cell harvest and immunoprecipitation of whole-cell lysates with antibodies specific for ER α . Immunoprecipitates were resolved by SDS-10% PAGE and processed by immunoblot analysis using antibodies specific for DBC-1 or ER α as indicated by arrows. Results are representative of at least three independent experiments.

erones and DBC-1 by coimmunoprecipitation analysis using MCF-7 whole-cell lysates. Whereas unliganded ER α immunoprecipitates included not only DBC-1 but also HSP90 (data not shown), DBC-1 immunoprecipi-

tates included unliganded ER α but neither HSP90 nor the immunophilin cyclophilin 40 (CYP40) (Fig. 2A). Thus, DBC-1 is not a component of the classical HSP90-based molecular chaperone complex. Subse-

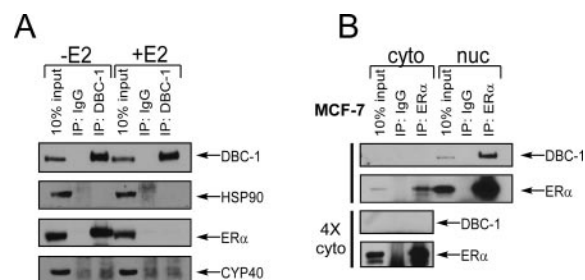


Fig. 2. DBC-1 and Unliganded ER α Associate in the Nucleus Independently of HSP90

A and B, Coimmunoprecipitation analysis. A, MCF-7 cells cultured in hormone-free medium for 3 d were treated without (–E2) or with (+E2) E2 (10^{-7} M) for 1 h before cell harvest and immunoprecipitation (IP) of whole-cell lysates with antibodies specific for DBC-1. Immunoprecipitates were resolved by SDS-10% PAGE and processed for immunoblot analysis with antibodies specific for DBC-1, HSP90, ER α , or CYP40 as indicated by arrows. Results are representative of at least three independent experiments. B, MCF-7 cells cultured in hormone-free medium for 3 d were fractionated into cytoplasmic (cyto) and nuclear (nuc) extracts. Equivalent amounts of each extract were immunoprecipitated with antibodies specific for ER α . Immunoprecipitates were resolved by SDS-10% PAGE and processed for immunoblot analysis with antibodies specific for DBC-1 or ER α as indicated by arrows. Note that an additional immunoprecipitation containing four times the amount of cytoplasmic extract (4 \times cyto) failed to yield a detectable amount of DBC-1 in either the input or immunoprecipitate. Results are representative of at least three independent experiments.

quently, we sought to identify the subcellular pool of unliganded ER α in specific association with DBC-1 by coimmunoprecipitation analysis using fractionated MCF-7 cell lysates. ER α /DBC-1 complexes were found exclusively in the nuclear fraction (Fig. 2B), thus revealing that unliganded ER α is distributed among at least two distinct protein complexes in human breast cancer cells: a cytosolic HSP90-based molecular chaperone complex and a nuclear DBC-1-containing protein complex.

The DBC-1 N Terminus Interacts Directly with the ER α Hormone-Binding Domain *In Vitro*

To determine whether DBC-1 interacts directly with unliganded ER α and to map the reciprocal binding domains on each protein, we tested the ability of glutathione S-transferase (GST)-ER α derivatives to bind to full-length DBC-1 or DBC-1 truncation fragments produced by *in vitro* translation. DBC-1 bound most efficiently to GST-ER α derivatives 1–595 (full-length ER α) and 302–595 (ER α hormone-binding domain), although DBC-1 also exhibited weak binding to GST-ER α derivative 251–301 (ER α hinge region) (Fig. 3A). Reciprocally, GST-ER α 1–595 (full-length ER α) bound to the extreme amino terminus of DBC-1 (amino acids 1–150) (Fig. 3B). Thus, in the absence of ligand, the ER α hormone-binding domain can accommodate the DBC-1 amino terminus.

The DBC-1/ER α Interface Is a Novel Target of Antiestrogens

Antiestrogens are currently the most widely administered endocrine agents for the management of ER α -expressing breast cancers (29, 30). Mechanistically, antiestrogens competitively displace E2 from the ER α hormone-binding domain and either block ER α function or induce destabilization and degradation of ER α . Tamoxifen, a prototype of the former class, is a selective ER modulator with antiestrogenic properties in breast and the most widely administered antiestrogen in breast cancer therapy (29, 30). Among the latter class of antiestrogens, ICI 182,780 (Faslodex; fulvestrant) is a selective ER down-regulator and an effective second-line therapeutic agent used to treat breast cancers that have progressed on previous tamoxifen therapy (29–31). Because these compounds bind directly to the ER α hormone-binding domain, we examined the influence of each agent on the DBC-1/ER α interaction. To this end, we tested the ability of ER α -specific antibodies to coimmunoprecipitate endogenous DBC-1 present in MCF-7 and BG-1 cells cultured in the absence or presence of E2, tamoxifen, or ICI 182,780. Strikingly, we observed that, like E2, both tamoxifen and ICI 182,780 disrupted the DBC-1/ER α interaction, thus revealing the DBC-1/ER α interface to be a heretofore unrecognized target of these endocrine compounds (Fig. 4, A and B).

DBC-1 Is an ER α -Dependent Prosurvival Factor in Breast Cancer Cells

To examine the biological consequence of the DBC-1/ER α interaction in human breast cancer cells, we first established conditions for RNA interference (RNAi)-mediated DBC-1 depletion in MCF-7 cells. Strikingly, we observed that RNAi-mediated DBC-1 knockdown was accompanied by a significant reduction in the steady-state level of ER α protein but not ER α mRNA, suggesting that DBC-1 modulates ER α protein synthesis or stability (Fig. 5). Notably, DBC-1 knockdown preferentially reduced the steady-state level of unliganded, but not liganded, ER α protein, consistent with the possibility that DBC-1 may stabilize unliganded ER α by virtue of their direct physical association (Fig. 5).

Because DBC-1 is a direct binding partner and key determinant of steady-state ER α protein levels, we examined its role in ER α -dependent breast cancer cell proliferation and survival. RNAi-mediated DBC-1 depletion significantly reduced E2-independent, but not E2-dependent, MCF-7 cell proliferation, an observation concordant with the fact that DBC-1 preferentially binds to and modulates the levels of unliganded ER α (Fig. 6). Because transient DBC-1 knockdown cells experienced an initial (~2-fold) reduction in cell number on d 3 after small interfering RNA (siRNA) delivery followed by growth kinetics similar to control siRNA knockdown cells, we hypothesized that the influence

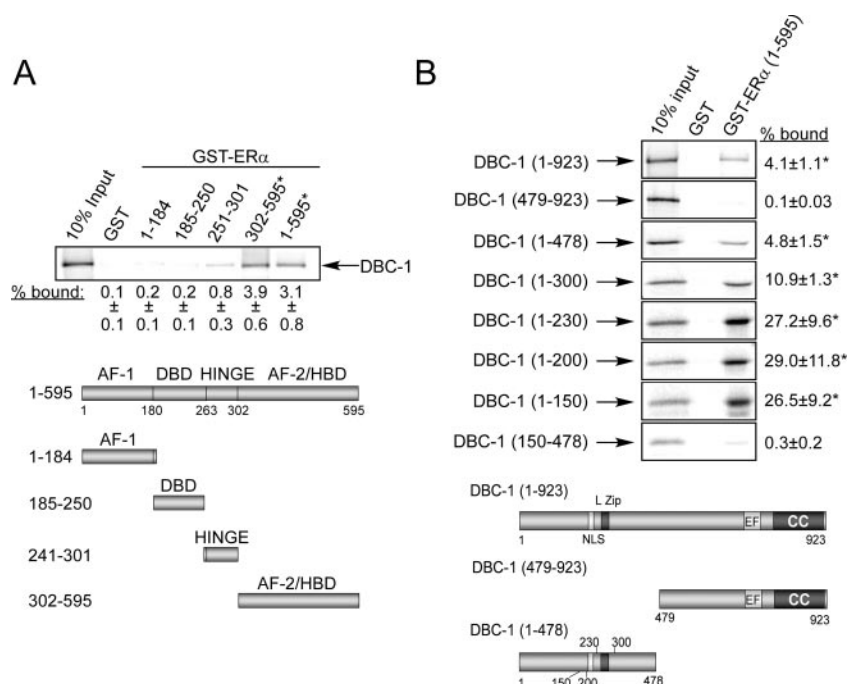


Fig. 3. The DBC-1 N Terminus Binds to the ER α Hormone-Binding Domain *in Vitro*

A and B, GST pull-down assays were performed using full-length *in vitro* translated DBC-1 and GST-ER α fragments (A) or *in vitro* translated DBC-1 fragments and GST-full-length ER α (B) as indicated. Numbers refer to amino acid coordinates. ^{35}S -labeled *in vitro* translated proteins were incubated with glutathione-Sepharose-immobilized GST derivatives, and bound proteins were resolved by SDS-12% PAGE before detection by PhosphorImager analysis. Input represents 10% of the ^{35}S -labeled *in vitro* translated proteins used in binding reactions. The amount of each DBC-1 derivative retained by GST-ER α (percentage bound) was quantified and expressed as a percentage of the total input. % bound refers to the average and SD of at least three independent experiments. *, $P < 0.05$, statistically significant binding values relative to GST alone. Note that DBC-1 binds primarily to GST-ER α derivatives 1–595 (full-length ER α) and 302–595 (ER α hormone-binding domain), whereas GST-ER α binds primarily to DBC-1 derivative 1–150 (N terminus). Schematic diagrams of ER α and DBC-1 indicate fragments used in binding reactions. AF-1, Activation function 1; DBD, DNA-binding domain; AF-2/HBD, activation function 2/hormone-binding domain; NLS, putative nuclear localization sequence; LZip, putative leucine zipper.

of DBC-1 silencing on ligand-independent cell proliferation may derive, at least in part, from an increase in apoptotic cell death. To address this question, we examined the influence of DBC-1 knockdown on the apoptotic fate of MCF-7 cells cultured in the absence of E2. Under these conditions, DBC-1 depletion increased the percentage of apoptotic cells from 6.2 to 12.8%, thus revealing an antiapoptotic function for DBC-1 in the absence of hormone (Fig. 7A). To determine whether DBC-1 promotes hormone-independent cell survival through its direct interaction with ER α , we also monitored the influence of DBC-1 knockdown on the apoptotic fate of MCF-7 cells cultured in the presence of E2, which disrupts the DBC-1/ER α interaction, or ICI 182,780, which not only disrupts the DBC-1/ER α interaction but also drastically depletes ER α protein levels. Notably, DBC-1 depletion had no effect on MCF-7 cell apoptosis in the presence of either E2 or ICI 182,780 (Fig. 7A). Furthermore, DBC-1 depletion did not enhance apoptosis of ER α -negative MDA-MB-231 breast cancer cells cultured in the absence of E2 (Fig. 7B). Together, these observations suggest that DBC-1 functions to promote E2-independent breast cancer cell survival in an ER α -dependent manner.

We note that we have also attempted to examine the influence of DBC-1 overexpression on breast cancer cell proliferation and survival; however, we have not been able to achieve overexpression of DBC-1 protein in ER α -expressing breast cancer cells, suggesting that DBC-1 expression levels are tightly regulated in this context.

DISCUSSION

Here we describe for the first time a biological function for DBC-1 in the modulation of ER α expression and survival activity in human breast cancer cells. Our identification of DBC-1 as a heretofore unrecognized determinant of steady-state ER α protein levels is a novel finding with implications for the regulation and function of ER α in normal and malignant breast epithelial cells. A compelling body of experimental, clinical, and epidemiological evidence suggests that dysregulation of ER α expression is a driving force in the initiation and progression of estrogen-sensitive breast tumors. ER α is the predominant receptor isoform ex-

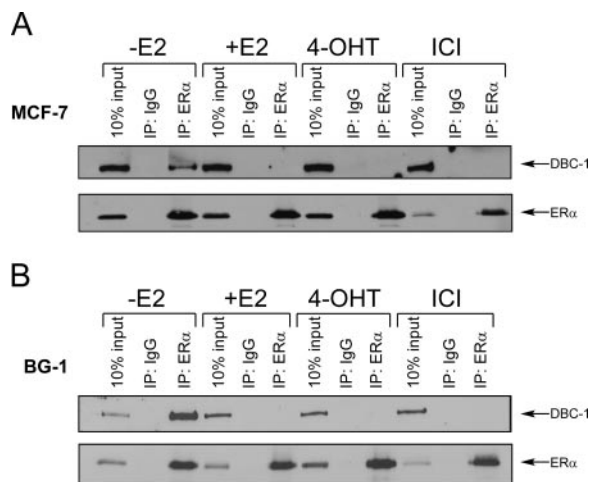


Fig. 4. Tamoxifen and ICI 182,780 Disrupt the Interaction between DBC-1 and ER α

A and B, MCF-7 (A) or BG-1 (B) cells cultured in hormone-free medium for 3 d were treated with vehicle (–E2), E2 (10^{-7} M; +E2), 4-hydroxytamoxifen (10^{-6} M; 4-OHT), or ICI 182,780 (10^{-7} M; ICI) for 1 h before cell harvest and immunoprecipitation (IP) of whole-cell lysates with antibodies specific for ER α . Immunoprecipitates were resolved by SDS-7.5% PAGE and processed for immunoblot analysis with antibodies specific for DBC-1 or ER α as indicated by arrows. Results are representative of at least three independent experiments.

pressed in breast cancer cells, and increased numbers of ER α -expressing cells as well as increased individual cell ER α content can be observed at the earliest stages of breast tumorigenesis (32, 33). It is thus likely that alterations in pathways leading to ER α synthesis and/or degradation underlie the dysregulation of ER α and its consequent manifestations, including enhanced proliferation in breast tumors. Therefore, revelation of the mechanism by which DBC-1 modulates ER α expression should yield important insight concerning the physiological regulation and, possibly, the pathological dysregulation of ER α in normal and malignant breast epithelial cells, respectively.

In this regard, previous work has revealed that unliganded ER α is sequestered by an HSP90-based molecular chaperone complex that protects the neosynthesized receptor from proteolytic degradation and primes it for ligand binding (26, 27, 34, 35). Our observation that HSP90 and CYP40 cannot be coprecipitated along with unliganded ER α by DBC-1-specific antibodies coupled with our finding that DBC-1 and unliganded ER α interact in the nucleus suggests that the cellular reserve of unliganded ER α is partitioned among at least two pools: one comprising cytosolic HSP90-based molecular chaperones and the other nuclear DBC-1. Notably, we observed that DBC-1 depletion preferentially reduced the steady-state level of unliganded ER α protein, suggesting the possibility that unliganded ER α is stabilized by its direct physical association with DBC-1. Thus, DBC-1 could function as a chaperone of ER α in the nucleus in

a manner analogous to that of HSP90 toward ER α in the cytosol. Additional studies will be required to elucidate the mechanism by which DBC-1 modulates ER α steady-state protein levels.

Several observations herein suggest a novel anti-apoptotic function for the population of unliganded ER α bound by DBC-1. First, apoptosis triggered by DBC-1 depletion in the absence of hormone was not observed in MCF-7 cells codepleted of ER α with ICI 182,780, nor in ER α -negative MDA-MB-231 breast cancer cells. These findings thus reveal an apparent DBC-1-dependent ER α requirement for suppression of apoptosis in the absence of hormone. Second, E2-mediated disruption of the interaction between DBC-1 and unliganded ER α abrogated the increase in MCF-7 cell apoptosis observed to accompany DBC-1 knock-down, suggesting that DBC-1-bound ER α functions to suppress hormone-independent apoptosis. We therefore speculate that a specific pool of unliganded ER α bound by DBC-1 may promote breast cancer cell growth and survival in the absence of hormone.

The underlying mechanism by which DBC-1 and ER α collaborate to promote hormone-independent breast cancer cell growth and survival remains to be established. As discussed above, DBC-1 could directly stabilize a pool of unliganded ER α dedicated to these functions. Whether or not DBC-1 additionally directly participates in ER α -regulated cell growth and survival processes is presently unknown. An intriguing possibility is that DBC-1 might function to mediate crosstalk between ER α and the NF- κ B survival pathway. Emerging evidence indicates that bidirectional molecular crosstalk between the ER α and NF- κ B pathway contributes to hormone-independent breast cancer cell growth and the development of antiestrogen resistance (36–40). Previously, DBC-1 has been linked physically to the NF- κ B pathway through a demonstrated interaction with I κ B kinase β (24), although our findings herein link DBC-1 physically and functionally to ER α . Possibly, DBC-1 could thus serve to stabilize and channel ER α toward functional interactions with the NF- κ B pathway. Future studies will be required to establish whether and how DBC-1-mediated crosstalk between the ER α and NF- κ B signaling pathways might contribute to hormone-independent breast cancer cell growth and survival.

Finally, our finding that ER α and DBC-1 collaborate to suppress apoptosis and promote hormone-independent breast cancer cell growth could have implications for breast cancer prognosis and/or treatment. It is generally believed that a balance between proliferation and apoptosis influences the response of breast tumors to hormonal therapy, and dysregulation of apoptotic signaling pathways has been suggested as a possible basis for treatment failure (29, 30, 41, 42). Accordingly, alterations in DBC-1 expression and/or activity could tip the balance between breast cancer cell growth and death; if so, DBC-1 could represent a novel biomarker of breast tumor response to endocrine therapy. In this regard, no published data

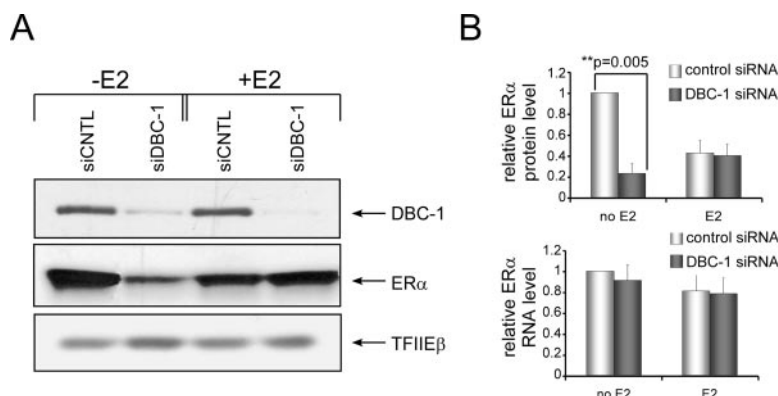


Fig. 5. RNAi-Mediated DBC-1 Suppression Is Accompanied by Reduced Steady-State Levels of Unliganded ER α

MCF-7 cells cultured in hormone-free medium for 3 d were electroporated with control (siCNTL) or DBC-1-specific (siDBC-1) siRNA (21 nM) as indicated. Electroporated cells were cultured without (–E2) or with (+E2) E2 (10^{-7} M) for an additional 3 d before cell harvest. **A**, Harvested whole-cell lysates were resolved by SDS-10% PAGE and processed by immunoblot analysis with antibodies specific for DBC-1, ER α , or TFIIIE β as indicated by arrows. Results are representative of at least three independent experiments. **B, Top**, Immune signals were quantified using a Kodak ImageStation 2000R. ER α protein levels were normalized to TFIIIE β and plotted relative to the ER α protein level in control siRNA cells cultured in the absence of E2, which was arbitrarily assigned a value of 1. Error bars represent the SD from the average of at least three independent experiments. **Bottom**, RNA was processed by quantitative RT-PCR analysis for the levels of DBC-1, ER α , and GAPDH mRNAs. ER α RNA levels were normalized to GAPDH levels and expressed relative to the level of ER α RNA in control siRNA cells cultured in the absence of E2, which was arbitrarily assigned a value of 1. Error bars represent the SD from the average of at least three independent experiments performed in duplicate.

currently exists concerning the relationship between DBC-1 and clinical response of breast tumors to endocrine therapy. Nonetheless, it would be useful to know whether overexpression or amplification of DBC-1 is linked to treatment failure. Furthermore, although DBC-1 is not deleted in most breast cancers, it would be of interest to know the hormone receptor and endocrine response status of the relatively small percentage of breast cancers that do harbor DBC-1 deletions. For example, might ER-positive patients

carrying DBC-1 deletions be underrepresented among the patient pool refractory to endocrine therapy? Answers to these and related questions should help to clarify the possible role of DBC-1 as a predictor of breast tumor response to endocrine therapy. From a possible therapeutic perspective, disruption of the DBC-1/ER α interface might provide a targeted means to reduce in breast tumors the number of hormone-refractory cells that arise through selection in response to prolonged endocrine treatment. Future experiments will be required to validate this hypothesis and further investigate the full spectrum of ER α -dependent and ER α -independent biological activities linked to DBC-1.

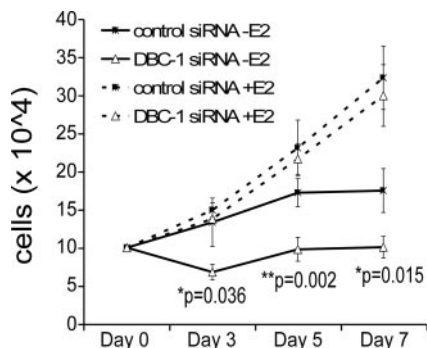


Fig. 6. RNAi-Mediated DBC-1 Depletion Inhibits Estrogen-Independent Proliferation in Human Breast Cancer Cells

MCF-7 cells cultured in hormone-free medium for 3 d were electroporated with control or DBC-1-specific siRNA (21 nM) as indicated and cultured without (–E2) or with (+E2) E2 (10^{-7} M). Culture medium was replaced every 2 d. Cell proliferation was monitored by counting with trypan blue exclusion for 7 d after electroporation. *P* values are compared with controls. Error bars represent the SD from the average of at least three independent experiments performed in triplicate.

MATERIALS AND METHODS

Expression Plasmids

pCS2+ER α was constructed by subcloning a *Bam*HI–*Bam*HI fragment carrying the full-length coding region of ER α cDNA from pG/ER(G) (provided by Dr. Dider Picard, University of Geneva, Geneva, Switzerland) (43) into pCS2+ (44). pACT-ER α was constructed by subcloning a *Bam*HI–*Bam*HI fragment carrying the full-length coding region of ER α cDNA from pCS2+–ER α into the pACT VP16 fusion vector (Promega, Madison, WI). GST-ER α (1–184), GST-ER α (185–250), GST-ER α (251–301), and GST-ER α (302–595) were gifts from Dr. Yi-Jun Zhu (Northwestern University, Evanston, IL) (45). GST-ER α (1–595) was generated by amplifying ER α by PCR and inserting it into the *Eco*RI site of pGEX-4T-3 vector (GE Healthcare, Little Chalfont, UK).

pSport1-DBC-1 was a clone obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung (www.rzpd.de) (RZPD clone DKFZp761O0817Q; KIAA1967). pCS2+DBC-1 was constructed by first amplifying the amino-terminal half of

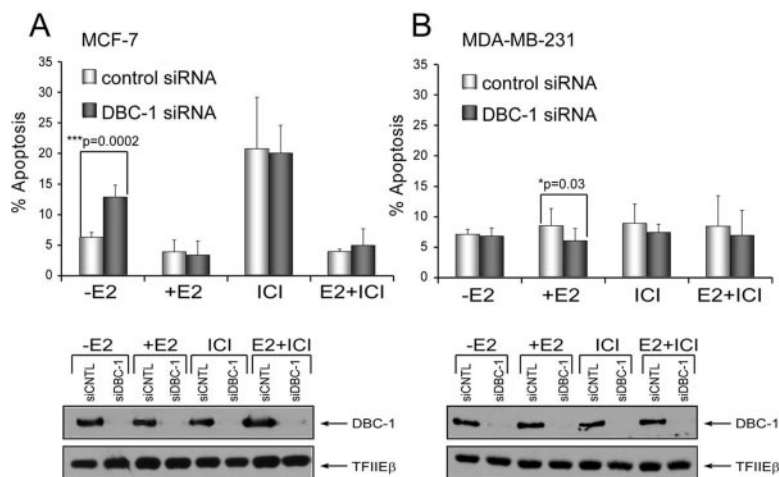


Fig. 7. DBC-1 is an ER α -Dependent Prosurvival Factor in Human Breast Cancer Cells

A and B, MCF-7 (A) or MDA-MB-231 (B) cells cultured in hormone-free medium for 3 d were electroporated with control or DBC-1-specific siRNA (21 nM) as indicated. Forty-eight hours after electroporation, cells were treated with vehicle (–E2), E2 (10^{-7} M; +E2), ICI 182,780 (10^{-7} M; ICI), or a combination of E2 and ICI 182,780 (E2+ICI) for an additional 24 h before cell harvest. *Top*, Harvested cells were stained with Annexin V-FITC and propidium iodide before quantification of apoptosis by flow cytometric analyses. *P* values are compared with controls. *Error bars* represent the SD from the average of at least three independent experiments performed in triplicate. *Bottom*, Cell lysates from representative apoptosis assays in A and B were resolved by SDS-10% PAGE and processed by immunoblot analysis with the indicated antibodies specific for DBC-1 or TFIIIE β as a loading control.

DBC-1 by PCR and inserting it into the *Clal/EcoRI* site of pCS2+.His6.FLAG, which yielded pCS2+.His6.FLAG-5' DBC-1.SphI. The carboxyl-terminal half of DBC-1 was amplified by PCR and then inserted into the *SphI/EcoRI* site of pCS2+.His6.FLAG-5' DBC-1.SphI to yield pCS2+DBC-1, which contains a STOP codon between the DBC-1 coding sequence and the His6.FLAG fusion. This construct was confirmed by sequencing. pCS2+.His6.FLAG-DBC-1 was generated by amplifying the carboxyl-terminal half of DBC-1 by PCR and then inserting it into the *SphI/EcoRI* site of pCS2+.His6.FLAG-5' DBC-1.SphI to create a version of DBC-1 fused to C-terminal 6XHis and FLAG tags. pCS2+DBC-1 amino-terminal fragments (1–478, 1–300, 1–230, 1–200, 1–150, and 150–478) were generated by amplifying fragments by PCR and inserting them into the *EcoRI/XhoI* site of pCS2+. pCS2+DBC-1 (479–923) was generated by amplifying the carboxyl-terminal half of DBC-1 by PCR and inserting it into the *EcoRI/XhoI* site of pCS2+. pBIND-DBC1 (1–478) was constructed by amplifying the amino-terminal half of DBC-1 by PCR and inserting it into the *XbaI/NotI* site of pBIND. pBIND-DBC-1 was constructed by amplifying the carboxyl-terminal half of DBC-1 by PCR and inserting it into the *XbaI/NotI* site of pBIND. pBIND-DBC-1 was constructed by subcloning an *XbaI/NotI* carboxyl-terminal fragment of DBC-1 from pBIND-DBC1 (479–923) into pBIND-DBC1 (1–478).

Reporter Plasmids

pG5luc, carrying five GAL4 DNA-binding sites upstream of the major late promoter of adenovirus driving expression of the firefly luciferase gene, was purchased from Promega.

Cell Lines and Culture Conditions

The HeLa (American Type Culture Collection, Manassas, VA), T-47D (American Type Culture Collection), MCF-7 (American Type Culture Collection), AmphiPack 293 (Clontech, Mountain View, CA), and MDA-MB-231 (American Type Culture Collection) cells were routinely cultured in DMEM (Invitrogen,

Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and penicillin-streptomycin-L-glutamine (Invitrogen). BG-1 cells, from Dr. Kenneth S. Korach (National Institute of Environmental Health Sciences, Research Triangle Park, NC) (46), were routinely cultured in DMEM/F12 (Invitrogen) supplemented as listed above. All cell lines except BG-1 and MDA-MB-231 cells were cultured at 37 C in a 10% CO₂ humidified chamber; BG-1 and MDA-MB-231 cells were cultured at 5% CO₂.

GST Pull-Down Assays

GST and GST fusion proteins were expressed in and purified from BL21-CodonPlus(DE3)-RIPL *Escherichia coli* (Stratagene, La Jolla, CA). Cells were grown at 37 C to A₆₀₀ of 1.0, and then isopropyl-1-thio- β -D-galactopyranoside was added to a final concentration of 0.5 mM. For GST, GST-ER α (1–184), GST-ER α (185–250), and GST-ER α (251–301), the cells were grown at 30 C for another 5 h. For GST-ER α (302–595), the cells were grown at 20 C for another 5 h. For GST-ER α (1–595), the cells were grown at 16 C for another 5 h. Cells were pelleted, washed once with PBS, and resuspended in lysis 250 buffer [50 mM Tris-HCl, 250 mM NaCl, 5 mM EDTA, and 0.1% Nonidet P-40 (NP-40)] supplemented with protease inhibitors (20 μ M antipain, 2 μ M pepstatin, 20 μ M leupeptin, and 2 μ g/ml aprotinin). Resuspended cells were subjected to one round of freeze-thaw, followed by sonication and clarification by centrifugation at 35,000 \times g for 30 min at 4 C.

Clarified GST lysates were bound to glutathione-Sepharose beads (GE Healthcare) for 45 min at 25 C, followed by washing four times for 5 min each with lysis 250 buffer containing 0.2% BSA and protease inhibitors. DBC-1 or fragments of DBC-1 were labeled with [³⁵S]methionine (TNT SP6 quick-coupled transcription/translation system; Promega) and incubated with immobilized GST proteins in PD buffer (50 mM Tris-HCl, 200 mM KCl, 5 mM MgCl₂, 5 mM EDTA, and 0.05% NP-40) for 2 h at 4 C. Binding reactions were washed with PD buffer three times for 5 min each at 4 C and subsequently boiled in 20 μ l of 1 \times Laemmli's sample buffer. Elu-

ates were resolved by SDS-12% PAGE and visualized by PhosphorImager analysis (GE Healthcare).

Mammalian Two-Hybrid Interaction Analysis

HeLa cells grown under hormone-free conditions for 2 d were plated at 1×10^5 cells per well in 12-well plates (Dow Corning, Corning, NY). After 24 h, the cells were transfected using FuGENE 6 (Roche, Indianapolis, IN) according to the recommendations of the manufacturer. In defining the ER α -DBC-1 interaction, transfection mixtures consisted of pCH110 (47), an internal control plasmid, expressing β -galactosidase under control of the simian virus 40 promoter (167 ng), pG5/luc reporter (167 ng), pACT-ER α (334 ng), and the various pBIND-DBC-1 constructs (334 ng), including pBIND-DBC-1, pBIND-DBC-1 (1–478), and pBIND-DBC-1 (479–923). pBIND empty vector was used as an appropriate control for interaction with pACT-ER α . pACT empty vector was used as an appropriate control for interaction with the various pBIND-DBC-1 constructs. After 48 h, cells were harvested and assayed for luciferase activity according to the guidelines of the manufacturer (Promega). Luciferase activity was corrected for the corresponding β -galactosidase activity to give relative activity. β -Galactosidase activity was assayed according to the instructions of the manufacturer (Tropix, Bedford, MA). Transfections were repeated a minimum of three times in duplicate. For experiments with ligand treatment, E2 (Sigma, St. Louis, MO) was added to cells at 10^{-7} M for 24 h before harvest.

For Western blot analysis, 48 h after transfection, whole-cell lysates were prepared in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% deoxycholate, 1% NP-40, and 0.1% SDS) supplemented with protease inhibitors and clarified by centrifugation. Equivalent amounts of lysates were boiled in Laemmli's sample buffer and resolved by SDS-10% PAGE. Proteins were analyzed by immunoblot using antibodies against GAL4-DBD (RK5C1; Santa Cruz Biotechnology, Santa Cruz, CA) and ER α (HC-20; Santa Cruz Biotechnology).

Coimmunoprecipitations

T-47D, MCF-7, or BG-1 cells were grown under hormone-free conditions for 3 d and treated without or with E2 (10^{-7} M), 4-hydroxytamoxifen (10^{-6} M; Sigma), or ICI 182,780 (10^{-7} M; Tocris, Ellisville, MO) for 1 h before cell harvest and coimmunoprecipitation. Whole-cell lysates were prepared in 0.5% NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40) supplemented with protease inhibitors and clarified by centrifugation. Nuclear and cytoplasmic extracts were prepared as described previously (48). Lysates were adjusted to binding buffer (50 mM Tris-HCl, 175 mM NaCl, 5 mM EDTA, 0.2% NP-40, and 10% glycerol, supplemented with protease inhibitors) concentration. Lysates were then subjected to immunoprecipitation with rabbit polyclonal anti-ER α (HC-20; Santa Cruz Biotechnology) antibody or mouse polyclonal anti-DBC-1 antibody [produced in our laboratory against recombinant DBC-1 (amino acids 475–923)] and protein A-Sepharose beads. Immune complexes were washed three times with binding buffer, boiled in Laemmli's sample buffer, and resolved by SDS-10% PAGE. Proteins were transferred to nitrocellulose membranes and visualized by using antibodies against DBC-1, ER α , HSP90 (rabbit polyclonal; Genetex, San Antonio, TX), CYP40 (rabbit polyclonal; Abcam, Cambridge, MA), appropriate peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA), and enhanced chemiluminescence detection (GE Healthcare).

DBC-1 Silencing by siRNA

To selectively knock down the expression of endogenous DBC-1 protein, an siRNA pool consisting for four different

target sequences was used (catalog no. 010427; Dharmacon, Chicago, IL). These RNA duplexes ($3 \mu\text{g}$ per 2×10^6 cells), as well as a negative control duplex that does not pair with any human mRNA (Dharmacon), were electroporated in MCF-7 or MDA-MB-231 cells using the cell line Nucleofector kit V (Amaya, Gaithersburg, MD). Immediately after control or DBC-1 siRNA electroporation, cells were seeded at a concentration of 1×10^6 per 60 mm plate. In all experiments, cells were allowed to grow for 3 d in phenol-red-free medium supplemented with 10% charcoal/dextran-treated FBS and without or with indicated chemical treatments. Cells were harvested 3 d after electroporation.

Western Blot Analysis

Three days after electroporation, whole-cell lysates were prepared in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% deoxycholate, 1% NP-40, and 0.1% SDS) supplemented with protease inhibitors and clarified by centrifugation. Lysates were boiled in Laemmli's sample buffer and resolved by SDS-10% PAGE. Proteins were analyzed by immunoblot using antibodies against DBC-1 (produced in our laboratory), ER α (HC-20; Santa Cruz Biotechnology), and TFIIIE β (C-21; Santa Cruz Biotechnology) as described previously. Quantification of Western blots was performed using the Kodak ImageStation 2000R (Eastman Kodak, Rochester, NY).

Quantitative Real-Time RT-PCR

Three days after electroporation, RNA was isolated from cells using TRIzol reagent (Invitrogen). RNA was reverse transcribed using random hexamers and Superscript III (Invitrogen) following the instructions of the manufacturer. Quantitative RT-PCR was performed using Absolute SYBR Green ROX Mix (ABgene, Rochester, NY) on an ABI PRISM 7900HT Fast real-time PCR system (Applied Biosystems, Foster City, CA). The gene-specific primers used were as follows: DBC-1, 5'-ATG TCC CAG TTT AAG CGC CAG-3' and 5'-CAA CCC CAA AGT AGT CAT GCA A-3'; ER α , 5'-CCA CCA ACC AGT GCA CCA TT-3' and 5'-GGT CTT TTC GTA TCC CAC CTT TC-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-CCT GTT CGA CAG TCA GCC G-3' and 5'-CGA CCA AAT CCG TTG ACT CC-3'.

Proliferation Assays

Three days before electroporation, cells were grown in phenol-red-free medium supplemented with 10% charcoal/dextran-treated FBS. Immediately after control or DBC-1 siRNA electroporation, cells were seeded at a concentration of 10×10^4 per well in six-well plates. In all experiments, triplicates of cells were allowed to grow for 7 d in phenol-red-free medium supplemented with 10% charcoal/dextran-treated FBS and without or with E2 (10^{-7} M) at 37 C and 10% CO $_2$. Cell viability was determined using the trypan blue exclusion assay, and viable cells were counted with the use of a hemacytometer. Proliferation assays were repeated a minimum of three times.

Apoptosis Assays

Three days before electroporation, cells were grown in phenol-red-free medium supplemented with 10% charcoal/dextran-treated FBS. Immediately after control or DBC-1 siRNA electroporation, cells were seeded at a concentration of 1×10^6 per 60 mm plate. In all experiments, cells were allowed to grow for 3 d in phenol-red-free medium supplemented with 10% charcoal/dextran-treated FBS and without or with E2 (10^{-7} M), ICI 182,780 (10^{-7} M; Tocris), or a combination of the two at 37 C and 10% CO $_2$. Seventy-two hours after electro-

poration, trypsinized cells (1×10^5) were stained with Annexin V-fluorescein isothiocyanate (FITC) (BD Pharmingen, San Diego, CA) and propidium iodide (Becton Dickinson, Franklin Lakes, NJ) according to the instructions of the manufacturer. Flow-cytometric analyses to quantify apoptosis were done in an FACSCalibur (Becton Dickinson). All Annexin V-FITC-positive cells were considered apoptotic. Apoptosis assays were repeated a minimum of three times.

Data Analysis

Statistical significance was assessed by comparing mean \pm SD values with Student's *t* test for independent groups. *P* \leq 0.05 was considered statistically significant.

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DBC-1 mediates endocrine resistant breast cancer cell survival

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Key words: DBC-1, ER α , breast cancer, endocrine resistance, apoptosis

Abbreviations: DBC-1, deleted in breast cancer 1; ER α , estrogen receptor α

Among a variety of etiological factors linked to breast cancer, the steroid hormone estrogen (17- β -estradiol; E2) has long been implicated in disease pathogenesis. Sustained exposure to E2 can induce and promote breast cancer, while estrogen ablation therapy or the administration of antiestrogens can oppose these effects.¹ The physiological effects of E2 in the breast are mediated by cognate receptors that are expressed as two structurally related subtypes, estrogen receptor α (ER α) and β (ER β).² ER α is the predominant receptor isoform expressed in breast cancer cells, and approximately 70% of breast cancer patients score positive for ER α at diagnosis.¹ ER α is therefore a dominant etiologic and valuable predictive factor with respect to breast cancer development and hormone sensitivity status. Endocrine therapy, which seeks to block ER-mediated mitogenic signaling through inhibition of E2 synthesis (aromatase inhibitors) or ER α transcriptional activity (antiestrogens) has emerged as one of the most important systemic therapies in breast cancer management; however, therapeutic resistance, either de novo or acquired during treatment remains a significant clinical roadblock to effective disease management.¹ The biological mechanisms underlying acquired endocrine resistance are incompletely understood and almost certainly multifactorial in nature. Nonetheless, the emergence of endocrine resistance is often coincident with a shift from hormone-dependent to hormone-independent control of ER α -regulated breast cancer cell growth and survival, possibly reflecting

bidirectional crosstalk between ER α and growth factor signaling pathways.² This knowledge has catalyzed the development and testing of signal transduction inhibitors as possible therapeutic agents to delay and possibly overcome endocrine resistance. However, clinical results to date have proved disappointing, suggesting the likely contribution of additional novel pathways.² Herein, we provide evidence that the deleted in breast cancer 1 gene product, DBC-1, mediates endocrine-resistant breast cancer cell survival. DBC-1 was originally identified during a genetic search for candidate breast tumor suppressor genes on a human chromosome 8p21 region frequently deleted in breast cancers.³ However, further analyses confirmed that DBC-1 expression is not substantially lost in cancers from any source.³ In fact, DBC-1 has been found to be upregulated in breast carcinoma versus normal breast tissue and in breast ductal carcinoma versus other cancers.⁴ Furthermore, DBC-1 is overexpressed in ER α -positive versus ER α -negative breast tumors.⁵ We previously reported that DBC-1 is a ligand-independent ER α -binding partner as well as an ER α -dependent pro-survival factor in human breast cancer cells.⁶ We showed the DBC-1 amino terminus binds directly to the ER α hormone-binding domain both in vitro and in human breast cancer cells in a strict ligand-independent manner. Notably, we showed that DBC-1 depletion inhibits estrogen-independent proliferation and promotes estrogen-independent apoptosis of ER α -positive, but not ER α -negative, breast cancer

cells. These findings establish a principal biological function for DBC-1 in the modulation of hormone-independent, yet ER α -dependent, breast cancer cell survival. DBC-1 has also been implicated in tumor necrosis factor (TNF) α -dependent apoptotic signaling and modulation of p53-mediated apoptosis through negative regulation of the NAD⁺-dependent deacetylase SIRT1.⁷⁻⁹ While the underlying basis for its pleiotropic activities remain to be clarified, it seems clear that DBC-1 plays an important contextual role in the balance between cell survival and death. Our observation that DBC-1 is a hormone-independent pro-survival factor in human breast cancer cells prompted us to examine its possible role in the acquisition of endocrine resistance. Thus, we examined the expression, ER α -interaction status, and function of DBC-1 in a three-stage MCF-7 cell-based model of acquired endocrine resistant breast cancer.¹ This model is based on the ER α -positive MCF-7 human breast cancer cell line, which is estrogen-dependent for growth and sensitive to the growth inhibitory actions of antiestrogens, including the selective estrogen receptor modulator (SERM) tamoxifen and the selective estrogen receptor downregulator (SERD) ICI 182,780 (fulvestrant). Long-term passage of MCF-7 tumor xenografts in ovariectomized mice led to derivation of the MCF-7/LCC1 (LCC1) cell line, which is estrogen-independent but antiestrogen-sensitive.¹ Subsequent long-term culture of LCC1 cells in the presence of ICI 182,780 produced the MCF7/LCC9 (LCC9) cell

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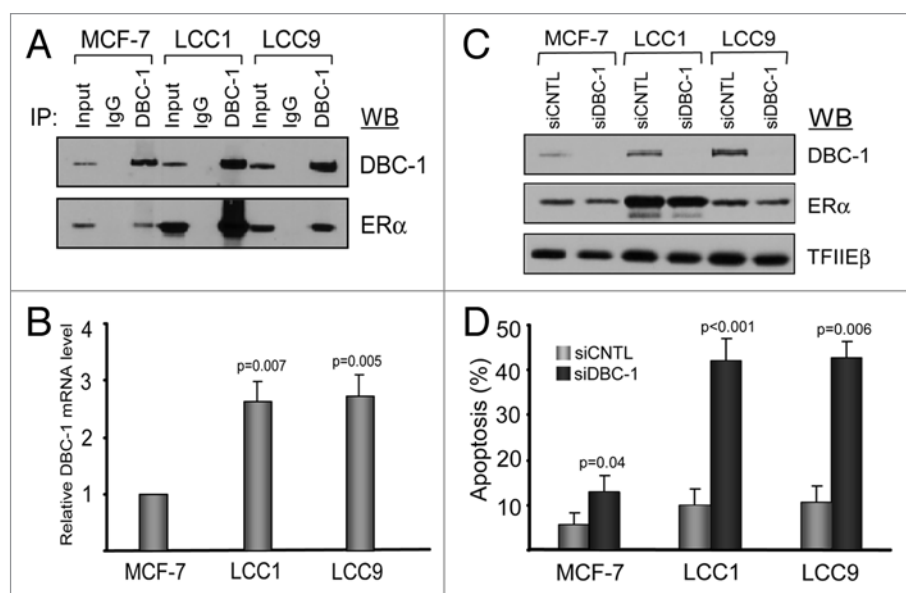


Figure 1. DBC1 mediates endocrine resistant breast cancer cell survival. (A) Whole cell extracts (500 μ g) from MCF-7, LCC1 and LCC9 cells cultured in hormone-free medium were subjected to immunoprecipitation (IP) with mouse IgG or mouse polyclonal antibodies specific for DBC-1 as indicated. Immunoprecipitates were resolved by SDS-10% PAGE and processed by western blot (WB) analysis using antibodies specific for DBC-1 or ER α . (B) RNA from MCF-7, LCC1 and LCC9 cells cultured in hormone-free medium was subjected to RT-qPCR analyses using primers specific for DBC-1. mRNA levels are expressed relative to the level in MCF-7 cells. Data represent the mean \pm SEM of at least three independent experiments performed in triplicate. p-values (Student's t test) correspond to differences between LCC1 or LCC9 cells and MCF-7 cells. (C) Whole cell extracts (25 μ g) from hormone-free cultures of MCF-7, LCC1 and LCC9 cells electroporated with control or DBC-1 specific siRNAs (20 nm) for 72 hrs as indicated were resolved by SDS-10% PAGE and processed by western blot (WB) analysis using antibodies specific for DBC-1, ER α or TFIIIE β (as an internal loading control). (D) MCF-7, LCC1 and LCC9 cells cultured in hormone-free medium were electroporated with control or DBC-1-specific siRNA (20 nm) 72 hrs prior to harvest. Harvested cells were stained with Annexin V-FITC and propidium iodide prior to quantification of apoptosis by flow cytometric analyses. Data represent the mean \pm SEM of at least three independent experiments performed in triplicate. p-values (Student's t test) correspond to differences between control and DBC-1-specific knockdowns. Validation of DBC-1-specific knockdown in a representative experiment is shown in (C).

line, which is estrogen-independent for growth, resistant to ICI 182,780, and cross-resistant to tamoxifen.¹ This model system, derived through stepwise selection of MCF-7 cells first to a low estrogen environment in vivo followed by long-term culture in the presence of an antiestrogen, conceptually mimics a clinical scenario (Phase II endocrine resistance) in which breast cancer patients undergo exhaustive hormonal therapy (first-line treatment with an aromatase inhibitor followed by second-line treatment with an antiestrogen) leading to the acquisition of an estrogen-independent and antiestrogen-resistant

tumor phenotype.¹⁰ Using this three-stage MCF-7 cell-based model, we observed that DBC-1 is upregulated during the progressive acquisition of endocrine resistance. Thus, steady state levels of DBC-1 protein (Fig. 1A and C) and mRNA (Fig. 1B) show incremental increases in MCF-7, LCC1 and LCC9 cell lines, respectively. Furthermore, the hormone-independent association between DBC-1 and ER α , previously observed in MCF-7 cells⁶ and reproduced here (Fig. 1A) is similarly conserved in both LCC1 and LCC9 cells (Fig. 1A). Finally, as observed previously,⁶ RNAi-mediated DBC-1 suppression

elicited an increase in apoptosis of MCF-7 cells cultured in the absence of hormone (Fig. 1D). Notably, however, we observed that DBC-1 suppression triggers a significantly more profound apoptotic response in estrogen-independent and antiestrogen resistant LCC1 and LCC9 breast cancer cells (Fig. 1D). Together, these findings establish DBC-1 as a critical and heretofore unknown determinant of endocrine resistant breast cancer cell survival. Previously, we showed that DBC-1 depletion promotes hormone-independent apoptosis of ER α -positive, but not ER α -negative, breast cancer cells in a manner reversible by endocrine agents that either disrupt the DBC-1/ER α complex (E2) or that reduce the level of ER α (ICI 182,780), suggesting that DBC-1 and ER α collaborate through direct interaction to promote hormone-independent breast cancer cell growth and survival.⁶ This observation, along with prior findings that ER α is required for estrogen-independent growth of LCC1 and LCC9 breast cancer cells¹¹ and our observation herein that the DBC-1/ER α interaction is conserved in these cells, leads us to hypothesize that the DBC-1/ER α complex helps drive antiestrogen resistance. Our findings could have important implications for breast cancer prognosis and treatment. First, our results point to DBC-1 expression as a possible biomarker of breast tumor response to endocrine therapy. Second, the DBC-1/ER α interface could represent a novel therapeutic target for pharmacological intervention in endocrine resistant breast cancer.

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